

09/22/98

JCS49 U.S. PTO

Please type a plus sign (+) inside this box ☐

# UTILITY PATENT APPLICATION TRANSMITTAL

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Attorney Docket No.	10806-64	Total Pages	32
First Named Inventor: Vincente Dias et al			
Title: Use of Beta Recombinase in Eukaryotic Cells, Especially for Transgenic Work			
Express Mail Label No.	EM382374597US		

## APPLICATION ELEMENTS

See MPEP chapter 600 concerning utility patent application contents

ASSISTANT COMMISSIONER FOR PATENTS  
ADDRESSED TO: Box Patent Application  
Washington, DC 20231

1. ☒ Utility Patent Application Transmittal [Total Pages: 2]
2. ☒ Specification, Claims and Abstract [Total Pages: 22]
3. ☒ Drawing(s) (35 USC 113) [Total Sheets: 5]
4. ☒ Oath or Declaration [Total Pages: 3]
  - a. ☒ Newly executed (original or copy)
  - b. ☐ Copy from a prior application (37 CFR 1.63(d)) (for continuation/divisional with Box 17 completed) [Note Box 5 below]
    - i. ☐ DELETION OF INVENTORS  
Signed statement attached deleting inventor(s) named in the prior application, see 37 CFR 1.63(d)(2) and 1.33(b).
5. ☐ Incorporation By Reference (useable if Box 4b is checked) The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied under Box 4b, is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference therein.

6. ☐ Microfiche Computer Program (Appendix)
7. ☐ Nucleotide and/or Amino Acid Sequence Submission (if applicable, all necessary)
  - a. ☐ Computer readable copy
  - b. ☐ Paper copy (identical to computer copy)
  - c. ☐ Statement verifying identity of above copies

## Accompanying Application Parts

8. ☒ Assignment Papers (cover sheet & document(s))
9. ☐ 37 CFR 3.73(b) Statement ☐ Power of Attorney (when there is an assignee)
10. ☐ English Translation Document (if applicable)
11. ☐ Information Disclosure Statement (IDS/PTO-1449)
  - ☐ Copies of Citations
12. ☒ Preliminary Amendment
13. ☒ Return Receipt Postcard (MPEP 503)
14. ☐ Small Entity Statement
  - ☐ Statement(s) filed in prior application, Status still proper and desired
15. ☒ Claim for Priority Under 35 U.S.C. §119

## 17. FEE CALCULATION

CLAIMS	(1) FOR	(2) NUMBER FILED	(3) NUMBER EXTRA	(4) RATE	(5) CALCULATIONS
	TOTAL CLAIMS (37 CFR 1.16(c))	26 -20 =	6	x \$22 =	\$ 132.00
	INDEPENDENT CLAIMS (37 CFR 1.16(b))	5 -3 =	2	x \$82 =	\$ 164.00
	MULTIPLE DEPENDENT CLAIMS (if applicable) (37 CFR 1.16(d))			+ \$ _____ =	
				BASIC FEE (37 CFR 1.16(a))	\$ 790.00
	Total of above Calculations =				\$1,086.00
	Reduction by 50% for filing by small entity (Note 37 CFR 1.19, 1.27, 1.28)				--
	SUBTOTAL =				\$1,086.00
	ASSIGNMENT RECORDATION FEE =				\$ 40.00
	TOTAL =				\$1,126.00

18. ☐ Please charge Deposit Account No. 04-1133 in the amount of \$ \_\_\_\_\_.
19. ☒ A check in the amount of \$1,126.00 is enclosed.
20. ☒ The Commissioner is hereby authorized to credit overpayments or charge the following fees to Deposit Account No. 04-1133:
- a. ☒ Fees required under 37 CFR 1.16
  - b. ☐ Fees required under 37 CFR 1.17.
  - c. ☐ Fees required under 37 CFR 1.18.

21. If a **CONTINUING APPLICATION**, check appropriate box and supply the requisite information:

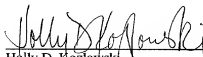
☐ Continuation ☐ Divisional ☐ Continuation-in-part (CIP) of prior application No. \_\_\_\_\_/\_\_\_\_\_

## 22. CORRESPONDENCE ADDRESS

☐ Customer Number or Bar Code Label or ☒ Correspondence Address Below

Name	Holly D. Kozlowski				
	Dinsmore & Shohl LLP				
Address	1900 Chemed Center				
	255 East Fifth Street				
City	Cincinnati	State	OH	Zip Code	45202
Country	USA	Telephone	513-977-8568	Fax	513-977-8141

Respectfully submitted,



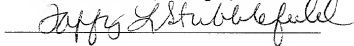
Holly D. Kozlowski  
 Registration No. 30,468  
 Attorney for Applicant(s)  
 Dinsmore & Shohl LLP  
 1900 Chemed Center  
 255 E. Fifth Street  
 Cincinnati, OH 45202  
 (513) 977-8568

### CERTIFICATE OF EXPRESS MAILING

"Express Mail" mailing label #: EM382374597US

Date of Deposit: September 22, 1998

I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to: Box Patent Application; Assistant Commissioner for Patents; Washington, DC 20231.



363447.01

Docket No. 10806-64

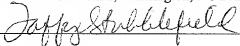
PATENT

**CERTIFICATE OF MAILING**

"Express Mail" mailing label #: EM382374597US

Date of Deposit: September 22, 1998

I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to: Box Patent Application; Assistant Commissioner for Patents; Washington, DC 20231.



**IN THE UNITED STATES PATENT & TRADEMARK OFFICE**

Applicant: Vincente Días et al : Paper No.:

Serial No.: To be assigned : Group Art Unit:

Filing Date: September 22, 1998 : Examiner:

For: **Use of Beta Recombinase in Eukaryotic Cells, Especially for Transgenic Work**

**PRELIMINARY AMENDMENT**

BOX Patent Application  
Assistant Commissioner for Patents  
Washington, DC 20231

Dear Sir:

Prior to calculation of the filing fee and first action by the Examiner in the present application, please amend the application as follows:

**In the Claims:**

Please cancel claims 1-26.

Please add the following claims 27-52:

--27. (NEW) A method for transgenic work in eukaryotic cells, comprising introducing prokaryotic beta recombinase and its specific target sequences in the eukaryotic cells.--

--28. (NEW) A method for controlling gene expression in eukaryots, comprising introducing prokaryotic beta recombinase and its specific target sequences in the eukaryots.--

--29. (NEW) A method for manipulating plant genomes in the generation of transgenic plants, comprising introducing prokaryotic beta recombinase and its specific target sequences in the plant genomes.--

--30. (NEW) A method for manipulating pathogenic and Gram positive bacteria, comprising introducing prokaryotic beta recombinase and its specific target sequences in the pathogenic and Gram positive bacteria.--

--31. (NEW) A method according to claim 27, wherein the eukaryotic cells are mammalian cells.--

--32. (NEW) A method according to claim 27, wherein site-specific intramolecular recombination between two *six* sites in eukaryotic cells is obtained.--

--33. (NEW) A method according to claim 32, wherein two or more different specific recombination events at a time are promoted.--

--34. (NEW) A method according to claim 32, wherein intramolecular reactions are exclusively mediated.--

--35. (NEW) A method according to claim 32, wherein the prokaryotic beta recombinase promotes the deletion of DNA sequences located between directly oriented *six* sites in mammalian cells.--

--36. (NEW) A method according to claim 32, wherein the prokaryotic beta recombinase promotes the inversion of DNA sequences located between inverted repeated *six* sites in mammalian cells.--

--37. (NEW) A method according to claim 32, wherein the prokaryotic beta recombinase promotes deletion of a DNA fragment laying between two directly oriented *six* sites.--

--38. (NEW) A method according to claim 37, wherein the prokaryotic beta recombinase promotes inversion of a DNA fragment laying between two inversely oriented *six* sites.--

--39. (NEW) A method according to claim 38, wherein the prokaryotic beta recombinase promotes deletion of a DNA fragment laying between direct repeated specific recognition sequences.--

--40. (NEW) A method according to claim 38, wherein the prokaryotic beta recombinase promotes inversion of a DNA fragment laying between inverted repeated specific recognition sequences.--



--50. (NEW) A method according to claim 43, wherein the *six* sites are integrated in the genome and wrapped on a nucleosome at several locations.--

--51. (NEW) A method for gene delivery in human gene therapy, comprising introducing prokaryotic beta recombinase and its specific target sequences in a human.--

--52. (NEW) A method according to claim 27 for development of transgenic animals.--

#### REMARKS

By the present amendment, claims 1-26 are cancelled and claims 27-52 are presented in order to set forth the invention according to customary U.S. patent practice. Claims 27-30 contain limitations from claims 2-4 and 6, respectively. Claims 31-40 contain limitations from claims 5 and 7-15, respectively, while claims 41 and 42 contain limitations from claim 16. Claims 43-52 contain limitations from claims 17-26, respectively. Since these claims do not involve any introduction of new matter, entry is believed to be in order and is respectfully requested.

Respectfully submitted,



Holly D. Kozlowski  
Reg. No. 30,468  
DINSMORE & SHOHL LLP  
1900 Chemed Center  
255 E. Fifth Street  
Cincinnati, Ohio 45202  
(513) 977-8568

Use of beta recombinase in eukaryotic cells, especially for transgenic work

5 The present invention relates to the use of prokaryotic recombinase in eukaryotic cells, especially for transgenic work in eukaryotic cells.

It also relates to the use of prokaryotic beta recombinase for intramolecular recombination in eukaryotic cells and for mediating exclusively intramolecular reactions.

10 The use of the gene coding for beta recombinase for catalyzing site-specific resolution (deletion) of DNA sequences when the two target sequences (DNA sequences containing *six* sites) in a plasmid (extrachromosomal target) introduced in the cell by transfection or when the target sequences are integrated in the genome as chromatin associated structures, at several locations is also disclosed.

15

## INTRODUCTION

Several methods have been developed allowing the manipulation of  
20 mammalian genomes in order to elucidate the relevance and function of particular genes of interest. Among them, the development of transgenic mouse strains and gene targeting technologies have turned out to be particularly useful (1, 2). These techniques have undergone a new advance with the characterization and application of site-specific recombinases (3).

25 Site-specific recombinases can be clustered into two major families. The first one (the Int family) comprises those enzymes that catalyze recombination between sites located either in the same DNA molecule (intramolecular recombination leading to resolution or inversion) or in separate DNA molecules (intermolecular recombination leading to integration) (4-7). The later property has been exploited to  
30 allow targeted insertion of specific sequences in precise locations (8, 9). The recombinases used for manipulating mammalian genomes are mainly the Cre and the



Flp proteins, which belong to the Int family (3). The target sequences for these enzymes, named *loxP* sites for the Cre enzyme and FRT for Flp, consist on a short inverted repeat to which the protein binds. The recombination process is operative through long distances (up to 70 kb) in the genome. Using these enzymes, several authors have reported site- and tissue-specific DNA recombination in murine models (10-13), chromosomal translocations in plants and animals (14-16) and targeted induction of specific genes (17). For instance, expression of Cre from the *lck* proximal promoter leads to specific recombination in thymus (10). The gene encoding DNA polymerase beta has been tissue-specifically deleted using the same strategy (11). In a different approach, the SV40 large tumor antigen has been specifically activated in the lenses of mice, resulting in tumours at that location and not in the rest of the animal (18). The Cre-*loxP* strategy has also been used in combination with inducible promoters, like the case of the interferon gamma inducible promoter, that was used to provoke gene ablation in liver with high efficiency and with less extent in other tissues (12). This powerful site-specific recombination system, however, only allow the induction of a reduced number of recombination event in the same genome. Since each recombination reaction leaves a target sequence for the recombinase in the genome at the crossover site, and because integrases (e.g. Cre and Flp) can catalyze intermolecular recombination, the whole process may lead to undesired chromosomal rearrangements. This general limitation has been proposed to be partially solved by the use of mutant versions of the *loxP* sequences (18).

The second family of recombinases includes those enzymes that catalyze recombination only when the sites are located in the same DNA molecule (resolution and / or inversion); they are collectively termed resolvases/invertases (19). These site-specific recombinases, which are constrained to intramolecular reactions, could have an advantage over the Int family of recombinases. beta recombinase, which belongs to the resolvases/invertases family, catalyses exclusively intramolecular deletions and inversions of DNA sequences located between two target sites for the recombinase, termed *six* (20-22). Each *six* site comprises 90 bp and is composed by two binding sites for the recombinase, termed I and II (20, 21, see Fig. 1). beta recombinase is

encoded by the beta gene of the Gram-positive-broad-host-range plasmid pSM19035 (22-25).

The earlier known Cre system appears to be limited in the sense that it allows the induction of a reduced number of recombination events within the same mouse genome. When a conditional knockout mouse is made using this system, a *loxP* site remains in the genome and constitutes a potential target for another recombination event of unknown consequences. This problem would be fully overcome using an exclusively intramolecular site-specific recombinase.

Figures:

Figure 1: A and B. Schematic representation of the site specific recombination mechanism mediated by IRAP and the plasmids used.

Figure 2. A - F. IRAP Expression

Figure 3. Functional expression of IRAP

Figure 4. IRAP-mediated site specific recombination.

## THE INVENTION

The scope of the invention is defined by the attached claims.

In the example the use of the prokaryotic site-specific beta recombinase for the manipulation of mammalian genomes is explored. The cloning and expression in eukaryotic cells of the gene coding for beta recombinase is described and its ability to catalyse site-specific resolution (deletion) of DNA sequences when the target sequences are either in a plasmid (extrachromosomal target) introduced in the cell by transfection, or integrated in the genome as chromatin associated structures, at several locations is shown. This activity is termed IRAP, for intramolecular recombination activity mediated by the beta protein. By target sequences is meant *site* or DNA sequences containing the natural *site* sites or modified versions that allow recombination activity

Our results show that beta recombinase is active in an eukaryotic environment, using the machinery/factors provided by the host cell and that beta recombinase can accurately and efficiently catalyse site-specific recombination in mammalian genomes. We show that the prokaryotic beta recombinase can be functionally expressed in eukaryotic cells and can promote the deletion of DNA sequences located between directly oriented target sites (intramolecular recombination activity mediated by the beta protein) in mammalian cells, IRAP. The availability of a tool like IRAP system, that can only catalyse intramolecular site-specific recombination, creates a wide variety of possibilities for the programmed modification of eukaryotic genomes, especially if combined with already-existing methods. For instance, a gene of interest could be engineered in order to have an exon or a group of exons flanked with *loxP* /FRT and *stop* sites. This would allow the study of gene product influence in two distinct tissues or in the same tissue in different developmental stages, through differential control of Cre/Flp and IRAP expression. Alternatively, two related genes could be inactivated at the same time or at two related stages. The use of inducible promoters would also allow more specific spatio-temporal control of the recombination event.

The possible applications and advantages of this new system is further elaborated under DISCUSSION and CONCLUSION

### Example 1.

## MATERIALS AND METHODS

### Plasmids and cloning

Plasmids pBT233 (23), pBT338 and pCB8 (20) and pLXSN, which carries the resistance marker for G418 (26), have been previously described. The eukaryotic expression vector pSV2 beta globin (27) was kindly provided by Dr. J. Ortin (CNB).

The expression plasmid pSV beta was constructed by PCR amplification of the coding sequence for the IRAP from the plasmid pBT233 . The primers used for PCR were:  
 betaUP 5'-GAGAGAAAGCTTGGTTGGTTGAAAATGGCT-3'  
 betaDO 5'-GAGAGATGATCAGTACTCATTAACATATCCC-3'

5

These oligonucleotides were engineered to contain *Hind*III and *Bcl*I restriction sites, respectively, which were used to clone the amplified gene in the pSV2 beta globin vector, following standard methods (28). Since *Bcl*I is sensitive to methylation, pSV2 beta globin plasmid was isolated from BZ101 (*dam*<sup>-</sup>) bacterial strain. The relevant structures are depicted in Fig. 1.

10

#### Culture and cell lines

Transient expression assays were performed in the simian COS-1 cell line kindly provided by Dr. J. Ortin. Stable clones with the DNA substrate for beta recombinase integrated at different sites of the chromosome were established in the murine cell line NIH/3T3 . Both cell lines were grown in Dulbecco's Modified Eagle Medium (DMEM) (Gibco-BRL, Grand Island, NY), supplemented with 10% foetal calf serum (Cultek, Madrid, Spain), 2 mM L-glutamin (Merck, Darmstadt, Germany) and the antibiotics streptomycin (0.1 mg/ml, Sigma, St. Louis, MO) and penicillin (100 U/ml, Sigma).

20

#### Transfection conditions and plasmid DNA extraction.

The transient expression experiments were performed in COS-1 cells by transfection with DEAE-dextran, as described (28). Cells were harvested 48 h after transfection and the extrachromosomal DNA was extracted using the method described by Hirt (29). In brief, cell pellets were lysed with SDS (Merck) and treated with proteinase K (Boehringer, Mannheim, Germany) at 37 °C. The genomic DNA was precipitated with 1 M NaCl (Merck). Upon centrifugation, the supernatant was phenol extracted and plasmid DNA precipitated with ethanol and resuspended in water for further experiments.

30

Stable cell clones with pCB8 DNA randomly inserted at different genome sites were obtained by electroporation in a Bio-Rad Gene Pulser (Hercules, CA) of  $2 \times 10^6$  NIH/3T3 cells at 220 V, 960 mF, with 20 mg of pCB8 DNA and a marker DNA coding for neomycin resistance at a ratio of 10:1. The neomycin resistant cells were selected with 1 mg/ml G418 (Sigma) for approximately two weeks. The stable clones obtained were analyzed in Southern experiments (28) or indirect immunofluorescence, as described.

#### Immunoblotting and immunofluorescence

Rabbit polyclonal antibodies against the purified beta recombinae were obtained by conventional techniques (28). beta recombinae was detected by indirect immunofluorescence or by SDS-PAGE followed by immunoblotting.

Transfected cells were grown on coverslips. After 48 h cells were fixed in methanol: acetone (1:1) at  $-20^\circ\text{C}$  for 5 min, air dried and rehydrated with PBS. Cells were then incubated with polyclonal anti- beta recombinae antibodies (1:5000 dilution) at room temperature for 30 min, washed three times for 5 min with PBS and incubated again with a fluorescein conjugated anti IgM antibody (DAKO, Glostrup, Denmark) for 1 h at  $37^\circ\text{C}$  in PBS. The cells were mounted in microscope slides and photographed in a fluorescence microscope.

For immunoblotting analysis, transiently transfected cells were harvested 48 h after transfection and lysed in RIPA buffer (137 mM NaCl, 20 mM Tris-HCl pH 8, 1 mM  $\text{MgCl}_2$ , 1 mM  $\text{CaCl}_2$ , 10 % glycerol, 1 % NP-40, 0.5 % sodium deoxycholate, 0.1 % SDS) (Merck). The lysed fraction was separated through SDS-PAGE, blotted onto nitrocellulose membrane (BIORAD), and incubated with polyclonal anti- beta recombinae antibodies, previously blocked with COS-1 total cell lysate (1/500 dilution). Peroxidase-conjugated anti-IgM antibody (DAKO, Glostrup, Denmark) was used as second antibody. Membranes were processed according to the ECL chemiluminescence detection kit (Amersham, UK).

# Analysis of recombination products

PCR was performed with the GeneAmp PCR System 2400 from Perkin Elmer (Foster City, CA), equipped with heating cover. Each reaction was carried out with 0.5 mg of genomic DNA or 1/10 of the Hirt preparation according to supplier's instructions. *Taq* polymerase (2.5 U) (Perkin Elmer) was added with Perfect Match PCR Enhancer (Stratagene, La Jolla, CA) after an initial denaturation (10 min, 94°C). Thereafter, the procedure (Touch-Down) was as follows: 80°C 2 min, 5 cycles of denaturation (94°C, 1 min) and annealing/extension at 73°C, 2 min, 5 cycles of 1 min at 94°C and 2 min at 70°C. This was coupled to 25 cycles of denaturation (94°C, 1 min), annealing (68°C, 30 sec) and extension (72°C, 2 min), and one additional cycle of extension at 72°C for 5 min. For the PCR analysis of the Hirt preparations we used the 16-mer reverse sequencing primer (# 1201) and the 17-mer universal sequencing primer (# 1211) from New England Biolabs (Beverly, MA).

The primers used for the PCR amplification of the Hirt preparations were unsuitable for the analysis of genomic DNA preparations (low  $T_m$ ). Therefore a new pair of primers was designed:

pBT338UP158, 5'-CCGCTCGTATGTTGTGTGGAAT-3'

pBT338DO802, 5'-TGGCGAAAGGGGATGTGCTG-3'

Southern analysis of the PCR products was performed by blotting the DNA separated through agarose gels onto nylon membranes (Amersham). Filters were hybridised at 42 °C in 250 mM phosphate buffer (pH 7.2), 50% formamide, 250 mM NaCl, 1 mM EDTA, 7% SDS, and washed in 1X SSC, 0.1% SDS, at room temperature for 30 min, at least twice. The washing temperature was increased when needed. The radioactive labelling of probes was performed with the Prime-It Random Primer Labelling Kit from Stratagene. Nucleotide sequences from the PCR bands of interest were determined by automated fluorescent sequencer and analyzed using Seq-Ed 1.0.3 software (both from Applied Biosystems Inc., Perkin Elmer).

## RESULTS

Expression of the prokaryotic beta recombinase in mammalian cells.

5       The coding sequence for beta recombinase was cloned in the pSV2 beta globin vector that directs expression from the SV40 virus early promoter. The resulting construction, pSV beta (Figure 1 B), was transiently transfected in COS-1 cells which express SV40 T-antigen. Under these conditions, the expression by plasmids that contain the early SV40 promoter - included in the pSV beta - is amplified. A control  
10       plasmid not containing beta recombinase gene, termed pSVc, was also generated.

      Transiently transfected cells were stained with specific antibodies raised against beta recombinase and viewed in a fluorescence microscope. The pSV beta transfected cells showed a strong speckled signal located specifically at the cell nucleus, as it can be seen in Figure 2, panels D and E. However, very faint staining  
15       was detected in the mock and control transfections (Fig. 2A, B and C). Similar results were obtained when the expression was tested in immunoblot (Fig. 2F). A specific 25 KDa band, corresponding to purified beta protein mobility (e), was developed by the anti- beta recombinase antibodies when COS-1 cells were transfected with the pSV beta plasmid (+) but not in the mock transfected cells (-). These results indicate that  
20       beta recombinase can be expressed in eukaryotic environments, showing strong avidity for the nuclear compartment.

      beta recombinase catalyzes site-specific recombination in transiently transfected mammalian cells.

25       Unlike Integrases with simple recombination sites, such as Cre and F1p, that require no additional protein factors (4, 5, 7), beta recombinase has a strict requirement for a chromatin-associated protein to mediate DNA recombination (20, 22). beta recombinase binds to the *six* sites and, with the help of a chromatin-associated protein, promotes strand exchange as depicted in Fig. 1A. Mammalian  
30       HMG1/HMG2 chromatin-associated protein can substitute, at least *in vitro*, for the

function of the analogous *B. Subtilis* or *E. coli* proteins , Hbsu and HU respectively (22).

To determine whether eukaryotic cells could account for this host factor, recombination activity due to beta recombinase was first checked by transient co-transfections in COS-1 cells with plasmids pSV beta (bearing the beta recombinase gene) and pCB8 (the substrate DNA containing two target sites for beta recombinase in direct orientation flanking the *xyE* gene, see Fig. 1B). Upon recombination, a derivative of pCB8 with a single *six* site should be obtained. The presence of this recombinant product can be easily monitored by PCR amplification of Hirt extracts, using primers complementary to the sequences located upstream of one of the *six* sites ( primer a in Fig.1B and Materials & Methods), and downstream of the second *six* site ( primer b in Fig.1B and Materials & Methods). In pCB8, these two primers hybridise to sequences located more than 2.7 Kb apart. Under our PCR conditions this fragment is not efficiently amplified. On the contrary, a 555 bp DNA segment should be amplified from the recombined product. A band of similar length should be obtained when using the same primers and plasmid pBT338 as template, that contains a single *six* site and was used as positive control (Fig.1B). Therefore, after transfection of the COS-1 cells (48h), the extrachromosomal fraction (Hirt extraction) of the cells was purified and the presence of recombination products analyzed by PCR. A clear amplified band of the expected length (555 bp) was observed only when both pCB8 and pSV beta plasmids were co-transfected ( see Fig 3); this band was absent when the two DNAs were transfected separately, or when pCB8 was co-transfected with pSVc, the negative control plasmid. The specificity of the amplified band was further confirmed by Southern hybridization (bottom panel) with a probe specific for the *six* site (see Fig 1A). A positive signal of the proper size was detected only in the positive control lane (Fig. 3, pBT338), and in the pCB8/pSV beta co-transfection sample. In lanes corresponding to transfections containing the pSV beta plasmid, the additional band of smaller size detected in the agarose gel was demonstrated to be non-specific, as it did not hybridise to the probe. These results show that beta recombinase is active in an eukaryotic environment, using the machinery/factors provided by the host cell.



beta recombinase promotes recombination in chromatin structures.

It has been described the need of supercoiled DNA as a critical condition for beta recombinase mediated deletions (22). To explore whether beta recombinase can promote DNA rearrangements when two *six* sites form part of the chromatin structure, we established NIH/3T3 cell clones in which the pCB8 construct was integrated at different locations within the mammalian genome. Several stable clones were analyzed by Southern hybridization. Five of them, each one carrying different number of copies (5-75, not shown) of the substrate plasmid, were chosen for transient transfection with the beta recombinase expression plasmid pSV beta. The presence of recombination products was analyzed by PCR of genomic DNA preparations using two primers (pBT338UP158 and pBT338LO802, see Materials and Methods; termed a' and b' respectively in fig. 1B) that should generate a 668 bp amplified fragment. The amplified DNA fragments were detected by Southern blot assays with a probe specific for the *six* site. In all cases, a band that hybridised with the probe (aprox. 660 bp) was detected in the pSV beta transfected samples (+) (see Fig. 4). This DNA fragment did not appear when plasmid pSV beta was not included in the transfection (mock transfection, - lanes).

The strength of the signal seemed to correlate with the number of copies of the target construction integrated in the chromosome (not shown), suggesting that recombination had occurred at most of the integrated target sequences. This result also suggests that the recombination efficiency is maintained regardless of the position of the target sequences within the chromatin.

The fidelity of the recombination mechanism was also confirmed by DNA-sequencing of the amplified bands in the case of clones 1 and 2 (data not shown). The regenerated *six* site (see Fig.1A) obtained after recombination was unaltered. All together, these data show that beta recombinase can accurately and efficiently catalyze site-specific recombination in mammalian genomes. It seems, therefore, that the chromatin structure provides a superhelical torsion suitable for beta recombinase mediated recombination. Although some of the *in vitro* features of beta recombinase activity are not still confirmed in mammalian environments (i.e. inversion, strict intramolecular recombination), we assume that they will be likely maintained in our

system. For this reason we define this recombination activity as IRAP for intramolecular recombination activity protein.

## DISCUSSION

The common genome manipulation techniques, including transgenesis and gene targeting, have opened a new path to understand a wide variety of mechanisms involving diverse genetic functions. However, the usefulness of these systems becomes limited when the expression or inactivation of a given gene has fatal effects on embryo development, (as an example, see ref. 11, 30) or when the lack of gene function can be bypassed or compensated by redundant mechanisms (31-33). Moreover, the effects of gene inactivation outside the tissue of interest are usually unknown and uncontrollable (34).

These problems have been partially overcome to some extent with the development and application of the site-specific recombination techniques (reviewed in 7) that allow spatio-temporal control of the targeting event. This is the case of the Cre-*loxP* and Flp-FRT systems (reviewed in 4 and 3). Nevertheless, although the Cre system has proven to be very useful, it seems to be limited in the sense that it allows only a reduced number of recombination event within the same mouse genome. When a conditional knockout mouse is made using this system, a *loxP* site remains in the genome and constitutes a potential target for another recombination event of unknown consequences. This problem would be overcome if an exclusively intramolecular site-specific recombinase can be used.

In this report we show that the prokaryotic beta recombinase can be functionally expressed in eukaryotic cells and can promote the deletion of DNA sequences located between directly oriented target sites (intramolecular recombination activity mediated by the beta protein) in mammalian cells, IRAP. The protein appears to have high avidity for the nuclear compartment since it was detected after transfection mainly in the nuclear region forming a very condensed and speckled pattern in indirect immunofluorescence. This behavior is similar to that observed for the Cre enzyme (13). Neither Cre nor beta recombinase present in their primary

sequence a canonical or bipartite nuclear localization motif (35, 36). Since they have access to the nuclear compartment, we assume that this localization occurs by diffusion through the nuclear membrane or following the transient disorganization of this membrane during mitosis.

5        Transient expression of IRAP by plasmid pSV beta was able to promote site-specific recombination between the two directly oriented *six* sites located in the substrate plasmid pCB8 when both plasmids were co-transfected into mammalian cells. As a result, the sequences comprised between the two target sites were deleted from the DNA substrate. The site-specific recombination product was detected by  
10    PCR amplification of the Hirt extracts and reassessed by southern hybridization of the amplified products. The presence of this recombination product was strictly dependent on the co-transfection of plasmids pSV beta and pCB8; no recombination products were observed when plasmids pSV beta or pCB8 were transfected separately. It is likely, therefore, that IRAP promotes strand exchange of an extrachromosomal DNA  
15    (pCB8 DNA) in the mammalian environment. Since *in vitro* recombination requires a chromatin-associated protein (25), we assume that this factor is provided by the host. Indeed, it is known that the mammalian HMG1 chromatin associated protein can efficiently stimulate *in vitro* beta-mediated recombination (22, 25). We have recently observed that chromatin-associated proteins from plants can also help beta  
20    recombinase to mediated DNA recombination ( S. Fernandez, K. Grasser, unpublished), suggesting that IRAP could be suitable for manipulation of plant genomes.

We have also investigated the ability of IRAP to act upon chromatin-integrated target substrates. For this, several stable NIH/3T3 clones were established bearing  
25    different numbers of copies (5-75) of the substrate plasmid pCB8 randomly integrated in the host chromatin. Transient IRAP expression led to the excision of the sequences comprised between the two directly oriented *six* sites; the recombination product was detected by PCR amplification from purified genomic DNA and Southern hybridization, and its identity was reassessed by direct DNA sequencing of the  
30    amplified product. In these assays, the amount of recombination products seemed to correlate with the copy number of the substrate DNA integrated into the genome,

suggesting a high efficiency of recombination and no or low position restriction effects.

The availability of a tool like IRAP system, that can only catalyse intramolecular site-specific recombination, creates a wide variety of possibilities for the programmed modification of eukaryotic genomes, especially if combined with already-existing methods. For instance, a gene of interest could be engineered in order to have an exon or a group of exons flanked with *loxP* /FRT and *six* sites. This would allow the study of gene product influence in two distinct tissues or in the same tissue in different developmental stages, through differential control of Cre/Flp and IRAP expression. Alternatively, two related genes could be inactivated at the same time or at two related stages. The use of inducible promoters would also allow more specific spatio-temporal control of the recombination event.

The IRAP system seems to have potential advantages over the already existing methods. Different authors have reported difficulties for the maintenance of *loxP* sites during the cloning procedures (37), though this seems not to be the case with the *six* sites. As the *six* site is larger than the 34-bp *loxP* site, the existence of potential cryptic sites is less likely, conferring greater target specificity on the recombination reaction. Such cryptic sites have been described for the Cre recombinase (38). Recently, the beta protein target site sequences necessary and sufficient to yield efficient recombination have been narrowed down to a 90 bp DNA segment that includes two recombinase binding sites (sites I and II); the crossing over point is located at the center of site I (39). It is unlikely that a DNA sequence of this size could be present fortuitously in a genome, therefore minimizing the possibilities of accidental chromosome reorganizations at serendipitous sites. In addition, the enzyme seems to have strong avidity for the nucleus, avoiding the need of introducing sequences for nuclear localization (37).

Preliminary results show that constitutive IRAP expression in NIH/3T3 is compatible with normal cell development (not shown). A transgenic mice model for the *in vivo* expression of the protein is under development. This would demonstrate the *in vivo* effectiveness of the IRAP-*six* system for the modification of precise targets.

As stated above, beta recombinase cannot promote intermolecular recombination *in vitro* or in bacterial cells. Since the potential for generating chromosomal reorganizations would thus be avoided, this tool could be used to promote two or more different specific recombination events at a time in the same animal model regardless of the constitutive expression of IRAP. Thus, the IRAP system offers new possibilities for the development of site-specific genome modifications.

## CONCLUSIONS

The development of new strategies for the *in vivo* modification of eukaryotic genomes has become an important objective of current research. Site-specific recombination has proven useful, as it allows controlled manipulation of murine, plant and yeast genomes. Here we describe that the prokaryotic beta recombinase is active in eukaryotic cells. beta recombinase has been functionally expressed in eukaryotic cell lines, demonstrating high avidity for the nuclear compartment and forming a clear speckled pattern when assayed by indirect immunofluorescence. In simian COS-1 cells, transient beta recombinase expression promoted deletion of a DNA fragment laying between two directly oriented specific recognition/crossing over sequences (*six sites*) located as an extrachromosomal DNA substrate. Such intramolecular recombination activity mediated by the beta protein in mammalian cells was termed IRAP. In stable NIH/3T3 clones bearing different copy numbers of the target sequences integrated at distinct chromosome locations, transient IRAP expression also promoted deletion of the intervening DNA, independent of its position and chromatin structure.

## FIGURE LEGENDS

Figure 1. Schematic representation of the site-specific recombination mechanism mediated by IRAP and the plasmids used in this study .

(A) The IRAP (shown as a dimer, open circles) interacts, in the presence of a host factor (HMG-1, shown as a monomer, open polygons) with two

identical copies of the *six* site (shaded and open arrows) to form a synaptic complex. This complex is resolved giving rise to a product and a circular intermediate, harboring the intervening sequences, that is finally lost. One full *six* site remains intact in the host cell. (B) The essential features of plasmid pSV $\beta$  and pCB8 and pBT338 DNA are indicated. The *six* site is shown schematically and sites I and II are highlighted. Orientation of the *six* sites is denoted by the sense of the shaded arrows. The coding region of the *xyE* gene, initially designed for expression in bacteria, is indicated. The hybridisation sites of the specific primers a/b and a'/b' pairs used for PCR amplification to detect the recombination products and the probe, used for specific hybridisation controls, are indicated.

Figure 2. IRAP expression in mammalian cells detected by indirect immunofluorescence and western blot. COS-1 cells, transfected with the indicated plasmids, were grown on glass coverslips. After 48 h, cells were fixed and stained as indicated. Panels A and B correspond to mock- and pSVc-transfected cells, respectively, incubated first with anti- $\beta$  recombinase polyclonal antibodies and developed with fluorescein-conjugated anti-rabbit IgG. Panels C, D and E show pSV $\beta$ -transfected cells. In panel C, the pSV $\beta$ -transfected cells were incubated first with preimmune rabbit serum, then developed as above. In panels D and E, showing two different fields of the pSV $\beta$ -transfected cells, they were incubated with polyclonal anti- $\beta$  recombinase antibodies and developed as above. Panel F. COS-1 cells, transfected as indicated, were harvested after 48 h and lysed as indicated in Materials & Methods. The whole cell extract proteins were resolved by SDS-PAGE, blotted onto nitrocellulose and analysed by western blot. The figure shows the autoradiography of a western blot incubated first with polyclonal anti- $\beta$  recombinase antibodies and developed with peroxidase-conjugated anti-IgM. C, purified  $\beta$  recombinase protein (10 ng); -, mock-transfected COS-1 cells;

and +, pSV $\beta$ -transfected COS-1 cells. The molecular mass of the band of interest is indicated.

Figure 3. Functional expression of IRAP assessed in transient transfection experiments. COS-1 cells were transfected with the indicated combinations of plasmids: pCB8, pSV $\beta$ , pSVc, pSVc + pCB8 and pSV $\beta$  + pCB8. Hirt extracts were obtained 48 h after transfection. The upper panel shows the result of PCR amplification from the Hirt extracts using the primer a/b pair indicated in Fig. 1B. Mock lane shows the negative controls of the PCR amplification, and lane pBT338 shows the positive control (100 pg of plasmid). The size marker lane corresponds to *Bst*EII-digested lambda DNA (500 ng). The lower panel shows the southern blot analysis of the agarose gel presented in the upper panel using a specific probe for the *six* site (see Fig. 1B). The position of the band of interest is highlighted.

Figure 4. IRAP-mediated site-specific recombination in chromatin-associated targets. Five NIH/3T3 clones (1-5) harboring several copies (approx. 75, 72, 12, 18 and 5, respectively) of pCB8 DNA integrated in different chromosomal locations were isolated and characterized. These clones were transiently transfected, either with (+) or without (-) plasmid pSV $\beta$ . Genomic DNA was purified 48 h after transfection and 500 ng of each sample were analyzed by PCR in the conditions described using the primer a'/b' pair (see Fig. 1B). One tenth of each sample was electrophoresed in 0.8% agarose gel, blotted onto nylon membrane and hybridised with an appropriate probe. Controls: (a) pBT338 (100 pg) and (b) pBT338 (100 pg + 500 ng of NIH/3T3 genomic DNA). The size marker corresponds to *Bst*EII-digested lambda DNA (500 ng). The size (0.66 kb) and position of the band of interest is indicated.

1. Brandon, E. P., Idzerda, R. L. and McKnight, G. S. (1995) *Curr Biol*, **5**, 625-34.
2. Brandon, E. P., Idzerda, R. L. and McKnight, G. S. (1995) *Curr Biol*, **5**, 758-65.
3. Kilby, N. J., Snaith, M. R. and Murray, J. A. (1993) *Trends Genet*, **9**, 413-21.
4. Sauer, B. (1993) *Methods Enzymol*, **225**, 890-900.
5. Dymecki, S. M. (1996) *Proc Natl Acad Sci U S A*, **93**, 6191-6.
6. Abremski, K. and Hoess, R. (1984) *J Biol Chem*, **259**, 1509-14.
7. Nash, H. A. (1996) in *Escherichia coli and Salmonella cellular and molecular biology*, ed. F. C. Neidhart, R. I. Curtis, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter and H. E. Umbarger (A. S. M. Press, Washington D. C.), pp. 2363-76.
8. Sauer, B. and Henderson, N. (1990) *The New Biologist*, **2**, 441-9.
9. Fukushima, S. and Sauer, B. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 7905-9.
10. DiSanto, J. P., Muller, W., Guy, G. D., Fischer, A. and Rajewsky, K. (1995) *Proc Natl Acad Sci U S A*, **92**, 377-81.
11. Gu, H., Marth, J. D., Orban, P. C., Mossmann, H. and Rajewsky, K. (1994) *Science*, **265**, 103-6.
12. Kuhn, R., Schwenk, F., Aguett, M. and Rajewsky, K. (1995) *Science*, **269**, 1427-9.
13. Orban, P. C., Chui, D. and Marth, J. D. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 6861-5.
14. Deursen, J. v., Fornerod, M., Rees, B. v. and Grosveld, G. (1995) *Proc. Natl. Acad. Sci. USA*, **92**, 7376-80.
15. Medberry, S. L., Dale, E., Qin, M. and Ow, D. W. (1995) *Nucleic Acids Res*, **23**, 485-90.
16. Osborne, B. I., Wirtz, U. and Baker, B. (1995) *Plant J*, **7**, 687-701.
17. Pichel, J. G., Lakso, M. and Westphal, H. (1993) *Oncogene*, **8**, 3333-42.
18. Sauer, B. (1996) *Nucleic Acids Res*, **24**, 4608-13.
19. Grindley, N. D. F. (1994) in *Nucleic Acids and Molecular Biology*, ed. F. Eckstein and D. M. J. Lilley (Springer-Verlag, Berlin), pp. 236-67.



20. Rojo, F. and Alonso, J. C. (1994) *J Mol Biol*, **238**, 159-72.
21. Rojo, F. and Alonso, J. C. (1995) *Nucleic Acids Res*, **23**, 3181-8.
22. Alonso, J. C., Weise, F. and Rojo, F. (1995) *J Biol Chem*, **270**, 2938-45.
23. Ceglowski, P., Boitsov, A., Chai, S. and Alonso, J. C. (1993) *Gene*, **136**, 1-12.
- 5 24. Rojo, F., Weise, F. and Alonso, J. C. (1993) *Febs Lett*, **328**, 169-73.
25. Alonso, J. C., Gutiérrez, C. and Rojo, F. (1995) *Mol Microbiol*, **18**, 471-8.
26. Miller, A. D. and Rossman, G. J. (1989) *Biotechniques*, **7**, 980-90.
27. Mulligan, R. C., Howard, B. H. and Berg, P. (1979) *Nature*, **277**, 108-14.
28. Maniatis, T., Fritsch, E. F. and Sambrook, J. (1982) *Molecular Cloning: A*  
10 *Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY), pp.
29. Hirt, B. (1967) *J Mol Biol*, **26**, 365-9.
30. Ioffe, E. and Stanley, P. (1994) *Proc Natl Acad Sci USA*, **91**, 728-32.
31. Schorle, H., Holtschke, T., Hünig, T., Schimpl, A. and Horak, I. (1991) *Nature*,  
**352**, 621-4.
- 15 32. Kühn, R., Rajewsky, K. and Müller, W. (1991) *Science*, **254**, 707-10.
33. Kopf, M., Lamers, M., Bluethman, M. and Kohler, G. (1994) *Nature*, **376**,  
339-45.
34. Dranoff, G., Crawford, A. D., Sadelain, M., Ream, B., Rashid, A., Bronson, R. T.,  
Dickersin, G. R., Bachurski, C. J., Mark, E. L., Whisett, J. A. and Mulligan, R. C.  
20 (1994) *Science*, **264**, 713-6.
35. Nath, S. T. and Nayak, D. P. (1990) *Mol Cel Biol*, **10**, 4139-45.
36. Robbins, J., Dilworth, S. M., Laskey, R. A. and Dingwall, C. (1991) *Cell*, **64**, 615-  
23.
37. Bergemann, J., Kuhlcke, K., Fehse, B., Ratz, I., Ostertag, W. and Lothar, H. (1995)  
25 *Nucleic Acids Res*, **23**, 4451-6.
38. Sauer, B. (1992) *J. Mol. Biol.*, **223**, 911-28.
39. Canosa, I., Rojo, F. and Alonso, J. C. (1996) *Nucleic Acids Res*, **24**, 2712-7.

## CLAIMS

- 5 1. Use of prokaryotic beta recombinase and its specific target sequences in eukaryotic cells.
2. Use of prokaryotic beta recombinase for transgenic work in eukaryotic cells.
- 10 3. Use according to claim 1 or 2 for controlling gene expression in eukaryotes.
4. Use according to claim 1 or 2 for manipulating plant genomes in the generation of transgenic plants.
- 15 5. Use according to claim 1 or 2 in which the eukaryotic cells are mammalian cells.
6. Use according to claim 1 or 2 for manipulating pathogenic and Gram positive bacteria.
- 20 7. Use according to any of the previous claim for site-specific intramolecular recombination between two *six* sites in eukaryotic cells.
8. Use according to claim 7 for promotion of two or more different specific recombination events at a time.
- 25 9. Use according to claim 7 for mediating exclusively intramolecular reactions.
10. Use according to claim 7 in which the prokaryotic beta recombinase promotes the deletion of DNA sequences located between directly oriented *six* sites in
- 30 mammalian cells.

11. Use according to claim 7 in which the prokaryotic beta recombinase promotes the inversion of DNA sequences located between inverted repeated *six* sites in mammalian cells.
12. Use according to claim 10 in which the prokaryotic beta recombinase promotes deletion of a DNA fragment laying between two directly oriented *six* sites.
13. Use according to claim 12 in which the prokaryotic beta recombinase promotes inversion of a DNA fragment laying between two inversely oriented *six* sites .
14. Use according to claim 13 in which the prokaryotic beta recombinase promotes deletion of a DNA fragment laying between direct repeat specific recognition sequences.
15. Use according to claim 13 in which the prokaryotic beta recombinase promotes inversion of a DNA fragment laying between inverted repeated specific recognition sequences.
16. Use according to claim 10, 11, 12 or 13 in which the specific recognition sequence is located as an extrachromosomal DNA substrate.
17. Use of the gene coding for beta recombinase for catalysing site-specific resolution of DNA sequences in an extrachromosomal target introduced into an eukaryotic cell.
18. Use of the gene according to claim 17 for catalysing site-specific resolution of DNA sequences when extrachromosomal target is a plasmid.
19. Use of the gene according to claim 17 or 18 in which the introduction is made by transfection.

20. Use of the gene according to any of claims 17 to 19 in which the resolution means deletion.
21. Use of the gene according to any of claims 17 to 19 in which the resolution means inversion.
22. Use of the gene according to any of claims 17 to 21 in which the DNA sequences are allocated between the *six* sites.
23. Use of the gene according to any of claims 17 to 22 when the *six* sites are integrated in the genome as chromatin associated structures.
24. Use of the gene according to any of claims 17 to 22 when the six sites are integrated in the genome and wrapped on a nucleosome, at several locations.
25. Use according to claim 1 to develop new techniques for gene delivery in human gene therapy.
26. Method for development of transgenic animals which includes the use of beta recombinase according to any of claims 1-21.

## ABSTRACT

The invention relates to the use of prokaryotic beta recombinase in eukaryotic cells, especially for transgenic work in eukaryotic cells.

It also relates to the use of prokaryotic beta recombinase for site-specific intramolecular recombination between two *six* sites in eukaryotic cells.

The use of the gene coding for beta recombinase for promoting the deletion of DNA sequences located between directly oriented *six* sites in mammalian cells and for catalysing site-specific resolution of DNA sequences in an extrachromosomal target introduced into an eukaryotic cell are also disclosed.

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20

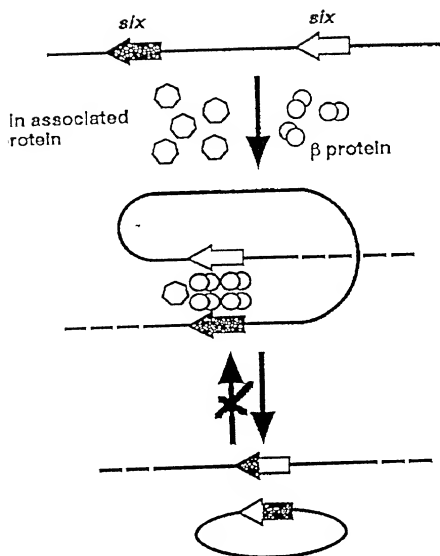


Figure 1A

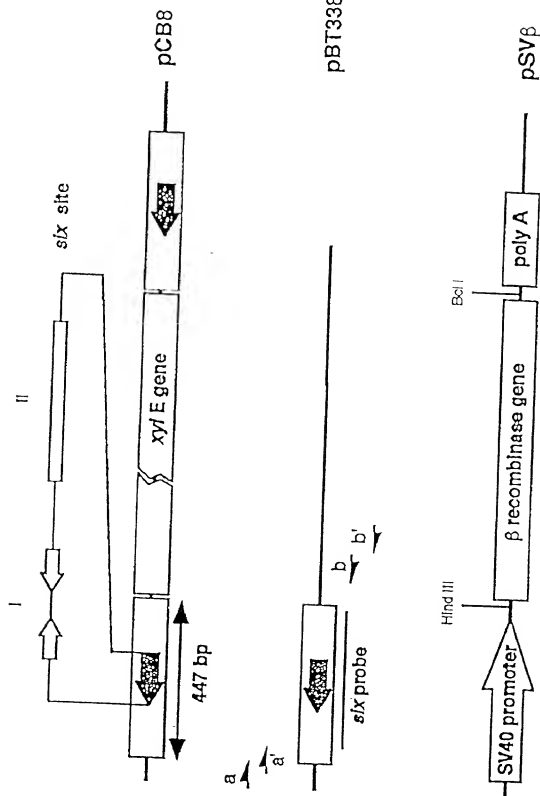


Figure 1B

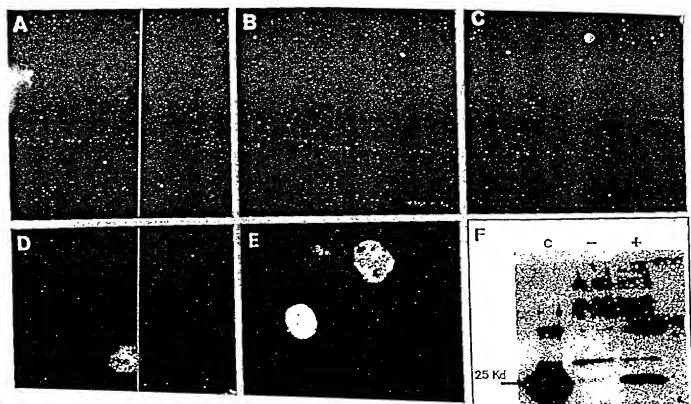


Figure 2



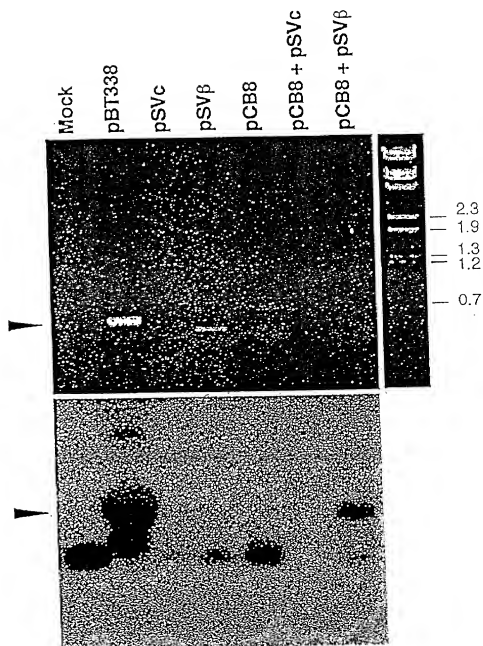


Figure 3

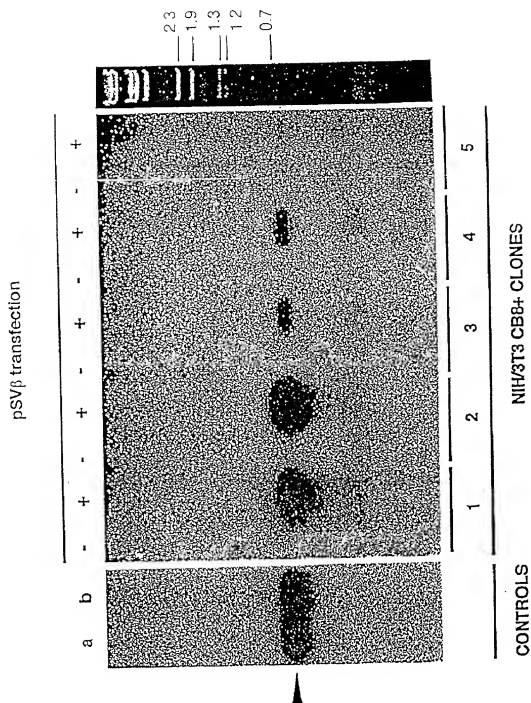


Figure 4

1787-03

**DECLARATION**  
**and**  
**POWER OF ATTORNEY**

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled Use of beta recombinase in eukaryotic cells, especially for transgenic work, the specification of which

☒ is attached hereto.

☐ was filed on \_\_\_\_\_ as  
Application Serial No. \_\_\_\_\_, and was  
amended on \_\_\_\_\_.

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby claim U.S. provisional application or foreign priority benefits under Title 35, United States Code, §119 of any U.S. provisional applications or any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior U.S. Provisional or Foreign Application(s)				
			Priority Claimed	
Number	Country	Day/Month/Year Filed	Yes	No
9703663-6	Sweden	8 October 1997	X	
60/062,994	U.S. Prov.	23 October 1998	X	

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulation, §1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

(Application Serial No.) (Filing Date)

(Status)  
(patented, pending,  
abandoned)

(Application Serial No.) (Filing Date)

(Status)  
(patented, pending,  
abandoned)

I hereby appoint Holly D. Kozlowski, Registration No. 30,468; Ronald J. Snyder, Registration No. 31,062; James D. Liles, Registration No. 28,320; Lynda E. Roesch, Registration No. 29,696; Phillip A. Rotman II, Registration No. 38,290; Jayadeep R. Deshmukh, Registration No. 34,507; Eric P. Jensen, Registration No. 37,647; Martin J. Miller, Registration No. 35,953; Victor C. Moreno, Registration No. 40,732; James C. Vago, Registration No. 40,855; Jackie A. Zurcher, Registration No. P42,251; and John V. Harmeyer, Registration No. P41,815, my attorneys, and Scott Barker, Registration No. P42,292, my patent agent, c/o Dinsmore & Shohl, 1900 Chemed Center, 255 East Fifth Street, Cincinnati, Ohio 45202 (513) 977-8200, with full power in each of them, of substitution and revocation, to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith.

The undersigned hereby authorizes the above-named U.S. attorneys to accept and follow instructions from Pharmacia & Upjohn AB as to any action to be taken in the Patent and Trademark Office regarding this application without direct communication between the undersigned and the aforementioned U.S. attorneys. In the event of a change in the firm or persons from whom instructions may be taken, the aforementioned U.S. attorneys will be so notified in writing by the undersigned.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

\*\*\*\*\*

Full name of sole or first inventor: Vincente Días

Inventor's signature

*V. Días*

*September, 14, 1998*

Date

Residence: Departamento de Inmunología y Oncología, Centro Nacional de  
Biotecnología, C.S.I.C., Campus de la Universidad Autónoma de Madrid, Cantoblanco,  
Citizenship: Spain ES-28049 Madrid, Spain.

Post Office Address: Same as Residence above.

Full name of second inventor: Fernando Rojo

Inventor's signature F. Rojo September 14, 1998 Date

Residence: Departamento de Biotecnología Microbiana, Centro Nacional de Biotecnología, C.S.I.C., Campus de la Universidad Autónoma de Madrid, Cantoblanco,  
Citizenship: Spain ES-28049 Madrid, Spain.

Post Office Address: Same as Residence above.

Full name of third inventor: Carlos Martínez Alonso

Inventor's signature Carlos Martínez Alonso September 13, 1998 Date

Residence: San Martín de Porres 14, ES-28035 Madrid, Spain

Citizenship: Spain

Post Office Address: Same as Residence above.

Full name of fourth inventor: Juan C. Alonso

Inventor's signature J. Alonso September 14, 1998 Date

Residence: Departamento de Biotecnología, C.S.I.C., Campus de la Universidad Autónoma de Madrid, Cantoblanco, ES-28049 Madrid, Spain

Citizenship: Spain

Post Office Address: Same as Residence above.

Full name of fifth inventor: Antonio Bernad

Inventor's signature A. Bernad September 15, 1998 Date

Residence: Departamento de Inmunología y Oncología, Centro Nacional de Biotecnología, C.S.I.C., Campus de la Universidad Autónoma de Madrid, Cantoblanco,  
Citizenship: Spain ES-28049 Madrid, Spain.

Post Office Address: Same as Residence above.

Full name of sixth inventor:

Inventor's signature \_\_\_\_\_ Date

Residence:

Citizenship:

Post Office Address: